# Interaction between the chromatin of *Beet curly top virus* and TFL2 protein

**Sara Hosseini**

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**Swedish University of Agricultural Sciences**
**Department of Plant Biology and Forest Genetics**
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# Interaktion mellan *Beet curly top* virus' kromatin och TFL2-protein

**Sara Hosseini**

**Supervisors:**

Anders Kvarnheden
Department of Plant Biology and Forest Genetics
Swedish University of Agricultural Sciences (SLU)
PO Box 7080, 750 07 Uppsala

Annika Sundås-Larsson
Department of Evolution, Genomics and Systematics
Physiological Botany
Uppsala University
PO Box Norbyvägen 18D, 752 36 Uppsala

**Examiner:**

Sarosh Bejai
Department of Plant Biology and Forest Genetics,
Swedish University of Agricultural Sciences (SLU)
PO Box 7080, 750 07, Uppsala

**Key words:**

Euchromatin: The lightly packed form of chromatin that is often under active transcription.
Geminiviruses: A devastating group of plant viruses which have single-stranded DNA genomes.
Histone: The proteins found in eukaryotic cell nuclei which package the DNA into nucleosomes.

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**Faculty of Natural Resources and Agricultural Sciences**
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# ABSTRACT:

*Beet curly top virus* (BCTV) is one of the most devastating DNA viruses, causing curly top diseases in a wide range of plants. This virus belongs to the family *Geminiviridae*, which replicate their circular single-stranded DNA genomes in the plant cell's nucleus through employing host replication factors. Transcription and replication are mediated by double-stranded DNA that associates with cellular histone proteins to form minichromosomes. Plants apply different defence mechanisms against geminiviruses. Among them, viral genome methylation is considered as an epigenetic defence, which targets the viral DNA cytosine residues and histone H3 at lysine 9 (H3K9), using a small RNA-directed methylation pathway. On the other hand, TERMINAL FLOWER 2 (TFL2) is a protein in Arabidopsis that is essential in the transition from vegetative to reproductive phase. This protein is associated with silencing of multiple euchromatin genes related to plant development, such as flowering and floral organ identity, through recognition of trimethylated H3 at lysine 27 (H3K27). This study was planned to investigate whether TFL2 plays a role in chromatin methylation of BCTV. Thus, in infection experiments, the *terminal flower 2* (*tfl2*) mutants of Arabidopsis were inoculated with BCTV to test if they show hypersusceptibility to the virus. However, no severe symptom was detected in these mutant plants in response to BCTV, suggesting that TFL2 does not play an important role in the defence against BCTV. To test the hypothesis that TFL2 binds to the geminiviral chromatin *in vivo*, TFL2-GFP transgenic Arabidopsis plants inoculated with BCTV, were tested in a chromatin immunoprecipitation (ChIP) assay. The method was planned based on immunoprecipitation of TFL2-GFP bound to the viral chromatin, using specific antibodies against GFP. The precipitated DNA obtained from the ChIP assay showed some amplification of the viral coat protein gene suggesting the possibility of an interaction between the TFL2 protein and BCTV chromatin.

# POPULAR SCIENCE:

*Beet curly top virus* (BCTV) is a leafhopper-transmitted virus, which causes curly top diseases in many plants. This virus belongs to the family *Geminiviridae*, which is the second largest family of plant viruses. Geminiviruses have a small, circular single-stranded DNA genome, which replicates in the plant cell's nucleus through a rolling circle mechanism. To counter these viruses, plants apply different defence mechanisms. Viral genome methylation is such a defence mechanism where a methyl group is added to the viral chromatin leading to virus suppression. TERMINAL FLOWER 2 (TFL2) is a protein in Arabidopsis that is essential for the transition from vegetative to reproductive phase. This protein is involved in silencing of many genes related to plant development, such as flowering time. The aim of this study was to investigate whether TFL2 plays a role in genome methylation of BCTV. Thus, *terminal flower 2* (*tfl2*) mutants of Arabidopsis were inoculated with BCTV to test if they show hypersusceptibility to the virus. However, no severe symptoms were detected in these mutant plants in response to the virus, suggesting that TFL2 does not play an important role in the defence against BCTV. In addition, to test the hypothesis that TFL2 binds to the chromatin of BCTV in plant cells, Arabidopis plants transformed with a TFL2-GFP construct were inoculated with BCTV and tested in a chromatin immunoprecipitation assay. The method was based on immunoprecipitation of a TFL2 viral genome complex, using specific antibodies against the GFP protein tag. Using polymerase chain reaction (PCR), the presence of viral genome in some of the precipitated DNA from the ChIP assay was proven, suggesting a possible interaction between TFL2 and BCTV chromatin. However, in this study, the ChIP analysis could not support the hypothesis that TFL2 interacts with BCTV chromatin and obviously the experiment needs to be repeated to verify if there is an association between this protein and geminiviral chromatin.

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# INTRODUCTION

## Family *Geminiviridae*:

Crops can be affected by different biotic and abiotic diseases and show a wide range of symptoms. Viruses are among the biotic agents that can attack many plants and in devastating cases reduce the yields even to zero and cause extreme losses in agriculture (Rojas, 2004). During the last two decades, among the different groups of plant viruses, the family *Geminiviridae* has emerged as a destructive group of pathogens which threaten the crop production, especially in tropical and subtropical regions (Varma et al., 2003).

This family is the second largest group of plant viruses. Geminiviruses infect a wide range of weeds and cultivated plants including both monocotyledonous and dicotyledonous plants such as maize, wheat, cassava, tomato, pepper, beet and cotton (Gutierrez, 2002; Rojas, 2004). Geminiviruses are transmitted by the insect vectors in a persistent manner and are capable of infecting the phloem cells. Plants can be affected by the infection in different ways. Photosynthesis is one of the physiological processes that is affected seriously, leading to a reduction in starch yield. Also, in some crops such as tomato, cotton and pepper, flowering and fruit formation can be disrupted by geminiviruses (Rojas, 2004).

Geminiviruses are characterized by a unique geminate (or twinned) shape particle, which is small in size (30 x 20 nm) and from which the group gets its name (Stanley et al., 1986). These viruses have monopartite or bipartite genomes of circular single-stranded (ss) DNA that replicates in the host cell nucleus. Each genome component is approximately 2.5-3.0 kb in length (Gutierrez, 2002). Based on genome structure, sequence, host range, tissue tropism, and insect vectors, geminiviruses have been classified into four genera: *Begomovirus*, *Mastrevirus*, *Topocuvirus* and *Curtovirus* (Rojas et al., 2005).

Mastreviruses (such as *Wheat dwarf virus*) are monopartite viruses, which are transmitted by leafhoppers and mostly infect monocotyledonous plants (Gutierrez, 2002; Park et al., 1999). The genus *Topocuvirus* contains only one member, *Tomato pseudo curly top virus*, which has a monopartite genome and is transmitted by treehoppers (Varma et al., 2003). Begomoviruses

(such as *Tomato yellow leaf curl virus* and *Bean golden mosaic virus*) are transmitted by whiteflies and infect dicotyledonous plants. These viruses can be monopartite or bipartite. The bipartite begomovirus genome consists of two ssDNA components referred to as DNA-A and DNA-B, which are similar in size. Finally, the leafhopper-transmitted curtoviruses (*Beet curly top virus*, BCTV) have a monopartite genome and infect dicotyledonous plants (Rojas et al., 2005).

Several factors including mutation, recombination, evolution of new variants of the viruses, appearance of efficient vectors, changing of cropping systems, weather events and movement of infected plants have lead to the emergence of these viruses to be considered as economically important problems (Varma et al., 2003).

Several interaction pathways between plants and these viruses have been already studied. Also, there are some factors which cause geminiviruses to be used as a tool to study DNA replication and regulation of gene expression in plants. Among those characteristics, having a small DNA genome and using double-stranded (ds) DNA for replication are the most remarkable ones (Rojas et al., 2005).

***Genome organization in the genera Mastrevirus, Topocuvirus and Begomovirus:***

The genome of geminiviruses contains 4 to 7 open reading frames (ORFs) in both the virion- and complementary-sense strands and also one or two intergenic regions (IR). The mastrevirus genome contains two virion-sense and two complementary-sense ORFs, with one long and one short IR. Topocuvirus has two ORFs in the virion sense and four in the complementary sense (Varma et al., 2003). In begomoviruses, DNA-A and DNA-B components have different nucleotide sequences except for 200 nucleotides, which are the same in the two DNAs and are named “common region” (CR) (Rojas et al., 2005). There are one or two virion-sense and four complementary-sense ORFs in DNA-A, while DNA-B contains one virion-sense and one complementary-sense ORF (Varma et al., 2003).

***Genome organization of Curtovirus:***

The monopartite genome of curtoviruses is 2.9–3.0 kb in size and contains four complementary-sense ORFs (Leftward ORFs), three virion-sense ORFs (Rightward ORFs) and one IR (Park et al., 1999; Varma et al., 2003) (Fig. 1). The virion-sense strand encodes three proteins including the capsid protein (CP), which encapsidates the virion-sense ssDNA genome and is important in virus movement and insect vector transmission, the movement protein (MP), and the V2 protein. The complementary sense encodes four proteins including the replication protein (Rep), a replication enhancer protein (REn/C3), the C2 protein that has a pathogenicity role in some hosts and the C4 protein which has an important effect on symptom development and cell-cycle control (Bolok Yazdi et al., 2008; Chen and Gilbertson, 2008; Gutierrez, 2002; Stanley, 2008). Generally, the proteins needed for regulation of transcription and replication are encoded by the complementary-sense strand, while the movement proteins and the protein with the structural functions are encoded by the virion-sense (Gutierrez, 2002). BCTV C4 is responsible for tumorigenic growth in infected plants that is caused by division of phloem parenchyma cells (hyperplasia) (Latham et al., 1998). The coding region of BCTV C4 overlaps with the *Rep* gene, but in a different reading frame, which shows the coordination of expression between two genes (Latham et al., 1997).

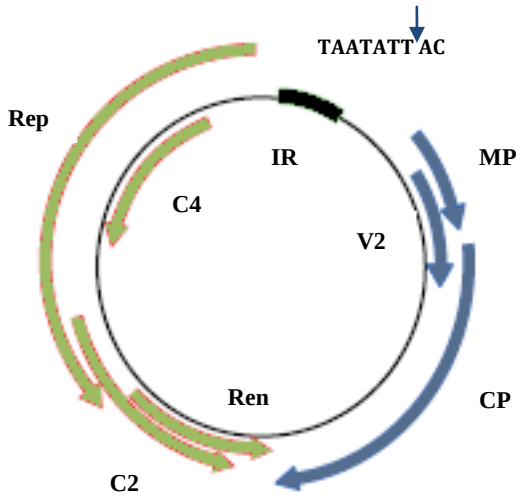


**Fig. 1.** The monopartite genome of *Curtovirus* (BCTV). Rep, the replication protein; Ren, the replication enhancer protein; MP, the movement protein; CP, the coat protein. IR is the non-coding intergenic region. TAATATTAC is the invariant sequence. The downward arrow (↓) shows the initiation site of rolling-circle amplification, adapted from Gutierrez, (2002).

## Replication:

Geminiviruses replicate their ssDNA genome in the nuclei of infected plant cells. Replication occurs through a rolling circle replication mechanism (RCR). In fact, after reaching the plant nucleus, the viral ssDNA genome converts into a covalently closed-dsDNA intermediate. Then, through the initiation site for RCR, which is located within the invariant 9-nt sequence TAATATTAC, the viral ssDNA starts to amplify. Finally, the replicated ssDNA will be encapsidated and transported to the neighbouring cells (Gutierrez, 2002).

Since geminiviruses have a small genome with a limited number of coding genes, they rely on host factors for replication. Their genome does not encode any DNA polymerase or other essential factors, except for the Rep protein (Hanley-Bowdoin et al., 2004). Rep (also named AL1, AC1 or C1) is the only necessary protein for the replication mechanism. It starts the RCR of the viral genome by binding to a specific DNA sequence. Also, it plays an essential role in reprogramming mature plant cells to support geminiviral replication (Arguello-Astorga et al., 2003). Some geminiviruses are limited to vascular tissues and replicate in vascular and bundle sheath parenchyma cells using the plant cell machinery. However, some geminiviruses can be found in differentiated cells of leaves, stem and roots. These mature cells have already finished the cell division cycle and do not any longer contain sufficient levels of DNA replication enzymes. So, it is necessary for geminiviruses to reprogramme their plant hosts and induce them to produce the required replication enzymes. Also, evidence indicates that in cultured cells, geminiviruses mostly replicate during S-phase, during which the crucial replication factors are available for the virus (Gutierrez, 2002). Thus, to provide suitable conditions for replication, geminiviruses must make the matured plant cells entering S-phase. They achieve this by interaction of the viral Rep protein with the plant homologue of the retinoblastoma-related tumor-suppressor protein (pRBR). Under normal conditions, pRBR binds to E2F, a protein which controls the entry into S-phase, and prevents its transcriptional activity. This interaction is disrupted by interference of viral Rep (Arguello-Astorga et al., 2003; Gutierrez, 2002; Hanley-Bowdoin et al., 2004; Rojas et al., 2005). The template which is transcribed in geminiviruses is the circular dsDNA. Transcription occurs bidirectionally through two divergent promoters which are located in the IR. These two promoters are separated by a non-transcribed region (Gutierrez, 2002; Hanley-Bowdoin et al., 1999).

## Curly top diseases:

BCTV (Stanley et al., 1986), type species of the genus *Curtovirus*, has been considered as a devastating pathogen of beet (*Beta vulgaris*) for about a century (Varma et al., 2003). Curly top viruses cause curly top disease (CTD) not only in sugar beet, but also in more than 300 plant species from 44 different families (Briddon et al., 1998). Pepper, bean, tomato, melon and spinach are among the susceptible hosts for these viruses. CTD has been known as an economically important disease in the Western United States since the early 1900s (Chen and Gilbertson, 2008). In the mid 1930s, resistant varieties of sugar beet were widely available in these regions. However, the use of resistant cultivars combined with insecticides can only decrease the incidence of disease, because even the most resistant cultivars can be damaged seriously if they are infected during early stages. BCTV destroyed about 80% of the chilli cultivation in southern New Mexico in the late 1990s (Varma et al., 2003). It is also a serious problem in other parts of the world, such as the Middle East and countries bordering the Mediterranean Sea (Strausbaugh et al., 2008). Different symptoms caused by curly top viruses include stunting, leaf curling, vein swelling and yellowing, crumpling, and hyperplasia of the phloem. Early infection causes early death in plants. The typical vein-swelling phenotype caused by curtovirus infection is associated with cell enlargement (hypertrophy) and deregulation of cell division (hyperplasia) in the phloem, correlated with expression of the viral C4 gene (Park et al., 2003; Piroux et al., 2007).

So far, six distinct species of curtoviruses have been characterized which all cause CTD. These species have been separated based on sequence variation of the genome, disease severity and differences in host range. Depending on their hosts, the severity of the disease is different. The genus *Curtovirus* includes BCTV (formerly the Cal/Logan strain), *Beet mild curly top virus* (BMCTV; formerly the Worland strain), *Beet severe curly top virus* (BSCTV; formerly the CFH strain), *Horseradish curly top virus* (HrCTV), *Spinach curly top virus* (SCTV) and *Beet curly top Iran virus* (BCTIV) (Bolok Yazdi et al., 2008; Chen and Gilbertson, 2008; Strausbaugh et al., 2008).

## Insect-virus relationship:

Curtoviruses are transmitted by the beet leafhopper (*Circulifer tenellus*) in a circulative persistent and non-propagative manner. Long-distance transmission of the viruses occurs through annual migration of leafhoppers. The insects lay eggs on annual weeds. The curly top viruses are acquired by nymphs from infected weeds. During spring, the adult vectors migrate to agricultural fields and transmit the virus to crops or weeds.

Feeding on phloem sap of infected plants, the insect acquires the virus as virions. Then, the virions pass through the food canal to the digestive system. Entering the hemolymph, the viruses circulate in the insect body and go into the salivary glands. During feeding, the viruses are transmitted with saliva to the new hosts. The latency period is short (around 4 hours) (Chen and Gilbertson, 2008).

## Plants defence mechanisms against geminiviruses:

### *Post-transcriptional gene silencing (PTGS):*

Plants use different mechanisms to counter viruses. RNA silencing, referred to as post-transcriptional gene silencing (PTGS), is an antiviral specific defence, which is used in both plant and animal cells. In this system, viruses are both inducers and targets. dsRNA is the genetic form, which induces RNA silencing. Then, short interfering dsRNA (siRNA) (21 to 26 nucleotides) is produced by activity of RNase III-like enzymes called Dicer or Dicer-Like. The RNA-induced silencing complex (RISC) guides the antisense strands of ssRNA to bind to the homologous ssRNA targets (usually mRNA) and degrades them. Among the factors involved in this mechanism, multiple Dicer-like (DCL) ribonucleases, RNA-dependent RNA (RDR) polymerases, and Argonaute (AGO) proteins are the important ones (Wang et al., 2005). Geminiviruses are also targeted by gene silencing and like most viruses they encode proteins that suppress this mechanism. AL2 and L2 are geminivirus proteins which play a role in suppression of silencing by interacting with adenosine kinase (ADK), a cellular enzyme that is involved in the maintenance of methylation (Roja et al., 2008; Wang et al., 2005).

## *Viral genome methylation:*

It has been shown that plants employ viral chromatin methylation as an epigenetic defence against geminiviruses. This methylation defence mechanism occurs through an RNA-directed methylation pathway, resulting in transcriptional gene silencing (TGS) or direct inhibition of replication and occurs in addition to PTGS, which leads to degradation of viral mRNAs (Buchmann et al., 2009; Roja et al., 2008) (Fig. 2). According to Roja et al. (2008), methylation of histone H3 at lysine 9 and cytosine residues of DNA plays an essential role in defence against geminiviruses. Arabidopsis plants with mutations in genes encoding ADK, cytosine or histone H3 lysine 9 (H3K9) methyltransferases, which are RNA-directed methylation pathway components, show hypersusceptibility to geminiviruses, suggesting that plants use methylation as an epigenetic defence mechanism against these viruses (Roja et al., 2008). However, it has been demonstrated that geminivirus AL2 and L2 proteins are able to suppress this host defence through interaction with ADK (Buchmann et al., 2009).

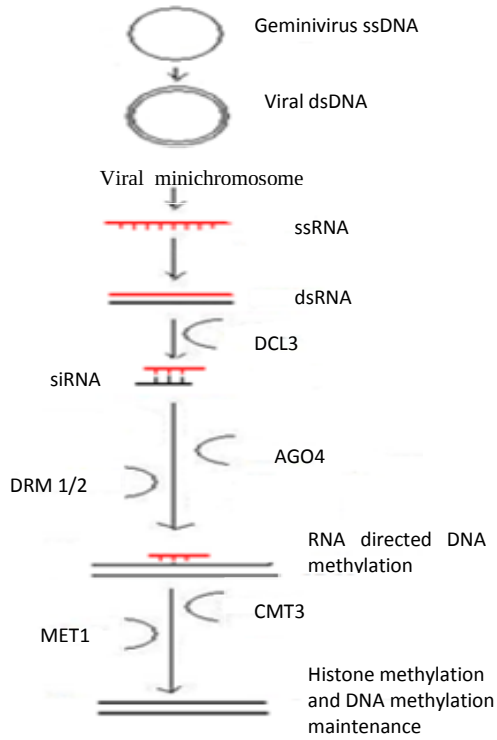


**Fig. 2.** A pathway for RNA-directed DNA methylation in Arabidopsis, described by Roja et al. (2008).

The target viral genome might be transcribed by an RNA polymerase IVa complex. The ssRNA is converted into dsRNA, which is cut by Dicer-like 3 (DCL3). The 24-nt siRNAs then are loaded into the complexes containing Argonaute 4 (AGO4) that lead the siRNAs to the homologous DNA sequences and methylation occurs by involvement of cytosine methyltransferases (e.g., DRM1/2). The methylation maintenance at CNG and CG sites requires the presence of the cytosine methyltransferases CMT3 and MET1, respectively.

**Chromatin structure:**

In eukaryotes, genomic DNA is packed into chromatin with histones, which are conserved basic proteins. This complex of DNA-protein consists of a repetitive nucleosomal structure. The nucleosome consists of 146 bp of DNA wrapped around a histone octamer (two copies of each of the four core histone proteins H2A, H2B, H3 and H4) (Eitokua et al., 2008). Each histone contains two functional and structural domains, an N-terminal region and the core domain (Eitokua et al., 2008). The N-terminal, which consists of 15-30 residues of amino acids and is referred to as tails, is continuously or temporarily modified (Kornberg and Lorch, 1999).

The structure of chromatin limits access of different enzymes and factors to DNA and blocks transcription while allowing access to linker DNA. Although the nucleosome appears positioned, it is a dynamic structure that can move rapidly. This mobilization is important in transcribed regions and also for promoter functions (Rando and Ahmad, 2007). Therefore, the maintenance and modification of chromatin structure have an essential role in gene regulation (Eitokua et al., 2008).

**Chromatin modifications:**

Generating open or closed chromatin structure, chromatin modifications, which occur through DNA or histone modifications, can activate or repress transcription. In open chromatin, transcription factors have more access to the genome, thereby transcription is active. Closed chromatin limits the accessibility of the genome to the general transcription machinery and represses transcription.

Histone modification alters primarily the amino acids located in the exposed N-terminal tails and subsequently the nature of the histone-DNA interaction, which leads to changes in the location of protein-binding sites. In histone modification, the modified amino acid, the type and the degree of modification (such as mono-, di- or trimethylation) are the factors determining whether the given modification is repressive or activating. Generally, acetylation and phosphorylation are the histone modifications which activate transcription, whereas methylation and ubiquitination can both activate and repress transcription (Pfluger and Wagner, 2007).

Histone modification occurs by histone modification enzymes such as histone acetyltranferases, histone methyltransferases and histone kinases. As an epigenetic gene regulation, histone modification affects gene expression and it also plays a role in formation of functional chromosomal domains (Eitokua et al., 2008).

**DNA and histone methylation:**

Heterochromatin is a region of the genome, which is characterized by tightly packed chromatin and less access to regulatory proteins. Since heterochromatin is inherited, there are two epigenetic marks, which lead to its maintenance during cell divisions. These marks are DNA methylation and histone H3 Lysine 9 (H3-K9) methylation.

DNA methylation is addition of one methyl group to the 5□ end of cytosine that as an epigenetic modification mechanism plays an important role in regulation of gene expression in plants and animals (Johnson et al., 2002). Cytosine methylation of DNA interfers with binding of some proteins (including transcription factors) and engages other proteins (Pfluger and Wagner, 2007; Zemach and Grafi, 2006).

In Arabidopsis, around one-third of the expressed genes show methylations within their transcribed regions. In plants, this mechanism, which is inherited through mitosis and often meiosis, occurs at symmetrical CpG and CpNpG sites as well as in non-symmetrical CpHpH (H = C, A or T) contexts. DNA methylation occurs by a mechanism known as RNA-dependent DNA methylation (RdDM), in which siRNAs are involved (Zemach and Grafi, 2006).

It has been demonstrated that, DNA methylation can influence the level of histone methylation. Also, DNA methylation can silence expression of genes in the absence of histone methylation, whereas H3-K9 methylation without DNA methylation is not sufficient for gene silencing (Johnson et al., 2002).

## TERMINAL FLOWER 2:

In Arabidopsis, the transition from vegetative to reproductive phase is regulated by many genes. Among them *TERMINAL FLOWER* 2 (*TFL2*/*LHP1*) (locus: AT5G17690) is necessary for the control of shoot meristem function. This regulation occurs through a double role of the protein. *TFL2* is active in response to the light signals affecting plant development. Furthermore, this gene is important for the maintenance of inflorescence meristem identity (Sundås-Larsson et al., 1998). *TFL2* is expressed in the meristem continuously throughout the vegetative, inflorescence and floral phases (Kotake et al., 2003).

Mutations in *TFL2* cause disruption in the normal photoperiodic flowering response as well as in inflorescence meristem development. Thus, *terminal flower 2* (*tfl2*) mutant plants show a range of developmental defects, including dwarfing, early flowering, conversion of the shoot apical meristem to a terminal flower, curled leaves, low fertility and reduced root growth (Nakahigashi et al., 2005; Sundås-Larsson et al., 1998; Turck et al., 2007). Also, *tfl2* mutant plants are unable to respond to heat-shock appropriately (Turck et al., 2007).

*TFL2*, which is located on chromosome 5 in the Arabidopsis genome, encodes a protein with homology to heterochromatin protein 1 (HP1) of animals and Swi6 of fission yeast and it is also referred to as Like heterochromatin protein 1 (Kotake et al., 2003). HP1 is a family of proteins with an evolutionary conserved N-terminal chromo domain (CD) and C-terminal chromo-shadow domain (CSD). HP1 is associated with gene silencing of heterochromatin genes. These protein molecules bind to methylated K9 on histone H3 and interact with chromatin through their CD domain (Hiragami and Festenstein, 2005).

*TFL2*, the only homolog of *HP1* in the whole Arabidopsis genome, contains 6 exons and encodes a protein consisting of 445 amino acids with a molecular weight of 48.6 kDa (Nilsson, 2007). Like its homolog, TFL2 is involved in repression of multiple genes. However, it is associated with silencing of euchromatin genes and not heterochromatin genes (Kotake et al., 2003; Nakahigashi et al., 2005).

It has been found that TFL2 represses transcription of many genes related to flowering time, such as *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS C* (*FLC*), as well as genes involved in floral organ identity, such as *AGAMOUS* (*AG*) and *APETALA* 3 (*AP3*). Since the expression of these genes is also regulated by proteins of the Polycomb repressive complex 2 (PRC2), it has been proposed that TFL2 interacts with PRC2 (Turck et al., 2007). Early flowering of *tfl2* mutant plants is due to over-expression of the *FT* gene, which is induced by an activator in response to the light signal. According to Takada and Goto (2003), the expression of *FT* is continuously repressed by TFL2 throughout development. Furthermore, TFL2 silences genes related to meiosis and seed maturation.

The molecular mechanism by which TFL2 silences euchromatin genes is still unclear. However, recent studies have indicated that the interaction of this gene with chromatin of numerous individual transcriptional units occurs through the recognition of histone H3 trimethylated at lysine 27 (H3K27me3). It has been shown that, *in vivo*, TFL2 associates with hundreds of genes marked by trimethylation of histone H3 lysine 27 (H3K27me3) rather than H3K9me2 or H3K9me3. Based on analysis of chromosome 4, it is predicted that TFL2 targets about 15% of the Arabidopsis genes (Turck et al., 2007).

## Chromatin immunoprecipitation (ChIP) method:

The epigenetic regulations refer to the heritable alterations of gene function without any changes in DNA sequences. DNA methylation and histone modifications are among these gene activating and repressive mechanisms, which occur through the interaction of DNA with various proteins. The chromatin immunoprecipitation (ChIP) method is a powerful tool to study protein-DNA interactions *in vivo* (Haring et al., 2007; Park, 2009; Saleh et al., 2008). This method is based on the cross-linking of proteins to DNA with formaldehyde which occurs through the reaction of formaldehyde with primary amines located on the amino acids of proteins and the bases of DNA molecules, leading to the formation of a covalent cross-link between the specific protein and the DNA fragment on which it is situated. Following crosslinking, the method consists of isolation of the chromatin complex, shearing the DNA fragments along with the linked proteins into a smaller size (around 500 bp) by ultrasound, immunoprecipitation of the DNA-protein complex using antibodies specific to the DNA-bound proteins, reversing the cross-link by heating, purification of the co-precipitated DNA and finally PCR amplification with specific primers to test whether the DNA of interest was precipitated (Fig. 3).

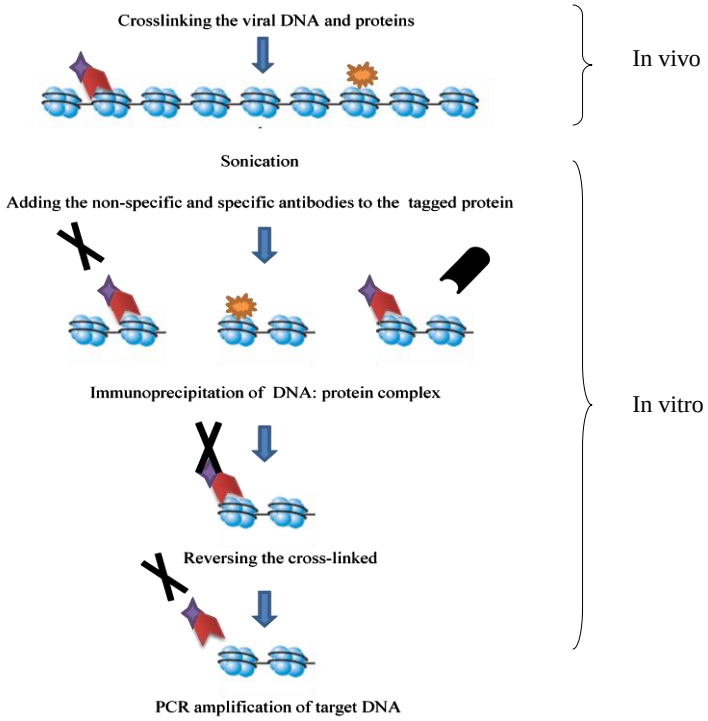


**Fig. 3.** The ChIP pathway used in this study, adapted from Cuthbert and Bannister.

# OBJECTIVE:

Plants use different defence mechanisms to counter geminiviruses. Among them, viral genome methylation as an epigenetic defence plays an important role in suppression of these viruses. RNA-directed DNA methylation leads to cytosine and H3K9 methylation of the viral genome through involvement of different essential methylation factors. On the other hand, TFL2, which is crucial in regulation of shoot meristem function, is associated with silencing of many euchromatin genes in Arabidopsis. This suppression occurs through recognition of H3 trimethylated at lysine 27 (H3K27) by TFL2. The objective of this study was to test whether TFL2 plays a role in methylation of the BCTV genome in Arabidopsis. Thus, the study was planned to study the response of *tfl2* mutants of Arabidopsis to BCTV infection. Also, to test the hypothesis that TFL2 can bind to the geminiviral chromatin, this protein-DNA interaction was studied *in vivo* using chromatin immunoprecipitation.

# MATERIAL AND METHODS:

**Plant material and growth conditions:**

The transgenic TFL2/ green fluorescent protein (GFP) plants used for the ChIP analysis had been produced in the Columbia (Col) accession of Arabidopsis. The construct of 35S::TFL2:GFP (locus tag: AT5G17690) had been prepared by fusing the GFP (S65T) ORF in-frame to the 3□ terminus of the *TFL2* cDNA, using a *Bam*HI linker in the pCGN transformation vector (Kotake et al., 2003). Arabidopsis Col ecotype was used as wild type control for the infection experiments. Two alleles of Arabidopsis *tfl2* mutants previously described by Sundås-Larson et al. (1998), *tfl2-1* and *tfl2-2*, were planted for studying the response to BCTV infection.

To repeat the inoculation experiments, Arabidopsis plants were planted three times. Each time, from each group of Arabidopsis (Col ecotype, TFL2/GFP transgenic, *tfl2-1* and *tfl2-2*) 40 plants were grown. After 2 days of stratification at 4°C, the seeds were sown in a 3:1 mixture of soil:vermiculite and grown at 20°C, in long-day (LD) conditions with a photoperiod of 18 hours light and 6 hours dark.

**Infectious clones:**

The BCTV isolate from California was first cloned (pBCT028), sequenced and characterized by Stanley et al. (1986). Then this genome was inserted into the binary vector pBin19 (Bevan, 1984) as partial repeats. The agroinfectious clone of BCTV was prepared conjugating the pBin19 construct into the AGL1 strain of *Agrobacterium tumefaciens* (Briddon et al., 1989). This clone was kindly provided by Dr. Margaret Boulton, John Innes Institute, UK. Ten-day-old cultures of *A. tumefaciens* on agar plates containing 50 μg/ml kanamycin as well as 50 μg/ml rifampicin were used for agroinoculation of plants (Boulton, 2008).

In the inoculation experiments, two types of negative controls were used: *Wheat dwarf virus* (WDV), for which Arabidopsis is not a host, and an empty vector. An agroinfectious clone of the wheat strain of WDV was previously constructed by Ramsell et al. (2009). *A. tumefaciens* AGL1 harbouring the infectious clone of WDV-[Enk1] in the binary vector pPZP201 was grown on agar plates containing 50 μg/ml rifampicin and 50 μg/ml spectinomycin. Also, *A. tumefaciens* with empty pPZP201 vector (Hajdukiewicz et al., 1994) was grown on agar plates with 50 μg/ml rifampicin.

**Inoculations:**

Inoculation experiments were carried out three times. Each time 40 TFL2-GFP transgenic Arabidopsis; 40 Arabidopsis Col ecotype as well as 40 *tfl2-1* and 40 *tfl2-2* plants were inoculated with BCTV as described by Boulton (2008). Agroinoculations of TFL2-GFP transgenic and Col ecotype Arabidopsis with BCTV were carried out around 30 days after germination, within 5 days of bolting (Roja et al., 2008). The mutants *tfl2-1* and *tfl2-2* were inoculated 15 days after germination (within 5 days of bolting), due to their earlier inflorescence time. Using a toothpick, a small amount of bacteria was collected directly from the ten-days-old plate culture and smeared onto the crown where the petiole joins to the stem. Then the inoculum was punctured into the stem by multiple stabs of an entomological needle. Also, from each group of plant, 10 plants were inoculated with WDV and 10 plants were inoculated with empty vector as negative controls. Inoculation with the agroinfectious clone of WDV and also with clones of *A. tumefaciens* AGL1 (with empty vector), were carried out in the same way as described above.

Furthermore, from each group of plants, 5 plants were stabbed multiple times by needle as a mock inoculation. Fifteen days after agroinoculation with BCTV, the transgenic and Col Arabidopsis plants showed symptoms of viral infection such as curling and necrosis. Symptomatic leaf tissue was harvested from transgenic, Col ecotype and mutant Arabidopsis plants 20 to 30 days post-inoculation.

**DNA extraction:**

DNA extraction was carried out according to a DNA miniprep protocol adapted from Edwards et al. (1991). This rapid method for preparation of plant genomic DNA for PCR analysis can be applied for DNA extractions from a variety of plant species. In addition to the short time required for a complete extraction, this method has the advantage of not using any phenol or chloroform (Edwards et al., 1991).

A small amount of leaf tissue was placed in an Eppendorf tube containing 1-2 glassbeads (4 mm) and 400 µl of Edward extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA). The samples were ground twice using a FastPrep-24 machine (MP Biomedicals Company) at 6,000 rpm for 40 seconds. After adding 0.5% SDS to the buffer, the tubes were vortexed for 5 seconds and left at room temperature for 5-10 minutes until all samples were extracted. The extracts were centrifuged at 13,000 rpm for 5 minutes. Three hundred µl of the supernatant was transferred to a new tube and mixed with an equal volume of isopropanol. The mixture was left at room temperature for 5 minutes. Following centrifugation at 13,000 rpm for 5 minutes, the supernatant was removed and after 15 minutes the dried pellets were dissolved in 50 µl MilliQ water. The results of the DNA extraction were checked by running an agarose gel. The concentration of extracted DNA was measured by NanoDrop and it was on average 150 ng/µl.

**Polymerase chain reaction (PCR):**

Samples were tested for presence of BCTV by PCR amplification of the *CP* gene using specific primers, which are designed to amplify a 496-bp fragment of all known curtovirus species: BCTV2-F 5′- GTGGATCAATTTCCAGACAATTATC-3′ and BCTV2-R 5′- CCCATAAGAG-

CCATATCAAACTTC-3′ corresponding to nucleotides 446 to 964 of BSCTV with accession U02311 in GenBank (Strausbaugh et al., 2008). Each time after inoculation of plants with BCTV, 15 samples of transgenic Arabidopsis, 15 samples of Col Arabidopsis and 10 samples of each *tfl2-1* and *tfl2-2* were selected randomly for testing.

PCR was run in a total reaction volume of 20 µl containing 1x PCR buffer (Applied Biosystems), 2.5 mM $MgCl_2$ (Applied Biosystems), 200 µM dNTPs (Fermentas), 0.2 µM primers, 250 units AmpliTaq Gold (Applied Biosystems) and 60 ng DNA. Amplification started by heating the samples to 95°C for 10 minutes followed by 34 amplification cycles. Each cycle consisted of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C. The last cycle was followed by 5 min at 72°C and then 12°C (Strausbaugh et al., 2008). Amplified products were analyzed by electrophoresis on an 1% agarose gel.

**Cloning:**

The *CP* fragments of the viral genome from 5 samples were purified using GeneJET™ PCR Purification Kit (Fermentas). The result of purification was checked by running an agarose gel. Then the purified *CP* fragments were ligated into pGEM-T Easy vector (Promega), using T4 DNA Ligase, according to the manufacturer's protocol. The ligation reactions in a total volume of 10 µl contained 5 µl of 2xligation buffer, 0.1 µg pGEM-T Easy vector, 1 µl of T4 DNA Ligase and 1.5 µl of PCR product and were incubated over-night at 4°C. To transform the plasmids into the bacterial cells of *Escherichia coli* DH5α, 50 µl of competent bacterial cells were mixed with 10 µl ligation product and incubated on ice for 30 minutes. After 20 seconds of heat shock at 37°C, the mix was incubated again on ice for 2 minutes. After adding 900 ml LB media, the cells were kept at 37°C under shaking for 90 minutes. Then, 100 µl of the cell culture was spread on LB plates containing 100 µg/ml ampicillin and X-gal. The plates were incubated at 37°C overnight. Three white colonies per plate were picked and grown overnight in 4 ml LB medium with ampicillin under shaking. The plasmid DNA was extracted using the Gene JET ™ Plasmid Miniprep Kit (Fermentas). For restriction, in order to check the presence of insert in the plasmid, the enzyme *Eco*RI was used following the Fastdigest (Fermentas). Three plasmids containing an insert of around 500 bp were sent to Macrogen/Korea for sequencing in both forward and reverse directions.

**Sequence analysis:**

To prove that plants had been inoculated with the BCTV-California isolate, the nucleotide sequence of the cloned PCR fragments were compared with sequences available in the GenBank database, using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Rolling circle amplification (RCA) / Restriction fragment length polymorphism (RFLP):**

To prove the presence of circular DNA of BCTV in the infected plants, the RCA/RFLP method was used. RCA allows amplification of the complete circular single-stranded DNA genome through a rolling-circular mechanism at a specific temperature (Demidov, 2005). RCA was carried out using the GE Healthcare kit. Five µl of sample buffer was mixed with 100 ng of extracted DNA and incubated at 95°C for 3 minutes. After the addition of 5 µl of reaction buffer and 0.2 µl of enzyme mix, the RCA reaction mix was incubated at 30°C for 18 hours. The reaction was stopped by incubation at 65°C for 10 minutes. To release the full-length unit of the genome, different restriction enzymes such as *Sac*I, *Age*I, *Aat*II and *Bgl*II, which have one unique cutting site in the BCTV genome, and also *Ssp*I and *Hpa*II, which have three cutting sites in the BCTV genome, were used. The restriction reaction in a total volume of 10 µl contained 2 µl of amplified DNA, 1 µl of buffer, 0.5 µl of enzyme and was incubated for 90 minutes at 37°C.

**Chromatin immunoprecipitation (ChIP) assays:**

The ChIP method was carried out based on the protocol described by Johnson et al. (2002) (Fig. 4). Plants were harvested 25 days after agroinoculation with BCTV. Symptomatic Arabidopsis inflorescence as well as leaf tissue (0.3 g) was cross-linked under vacuum for 20 minutes in 6 ml of buffer A containing 0.4 M sucrose, 10 mM Tris pH 8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors (1 µg/ml leupeptin and pepstanin, 0.5 µg/ml aprotinin) and 1% formaldehyde. The reaction was quenched by adding glycine to a final concentration of 0.1 M and continued for 10 minutes under vacuum. The plant tissue was then washed twice in MilliQ water and frozen in liquid nitrogen. Each sample (0.3 g) was ground and resuspended in 1 ml lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 10 mM PMSF, 0.01 µg/ml aprotinin, 1 µg/ml pepstatin and leupeptin). The DNA was sheared by sonication to obtain fragments of

approximately 250-1000 bp (8 times for 20 seconds at 12% effect of Sonicator ultrasonic processor, Misonix Company). After centrifugation for 10 minutes at 13,000 rpm, 10 µl of supernatant was saved to check the size of sonicated fragments. To preclear the supernatant of each sample, 50 µl of Protein A agarose beads (Santa Cruze Biotechnology) was washed 3 times in 1 ml ChiP dilution buffer (1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0 and 167 mM NaCl). To recover the beads, samples were centrifuged at 13,000 rpm for 1 min. Then the supernatant (chromatin), which was taken from each sample, was added to the prepared protein A agarose beads and precleared for 60 minutes at 4°C under slow rotation. After centrifugation at 3,000 rpm for 2 min, the supernatant was transferred to new tubes and 10 µl of IgG (as control precipitation) or 15 µl of GFP antibody (Upstate /Millipore) was added. After incubation overnight at 4°C with rotation, 70 µl protein A agarose beads, which were prepared as described in the preclearing step, were added and incubation continued for 4 hours. The beads were precipitated after 2 min centrifugation at 3,000 rpm at 4°C and the supernatants were kept as the input and stored at -20°C. The beads were washed twice in 1 ml lysis buffer, once in 1 ml LNDET (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8]) and 3 times in TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). Between each washing step, the beads were incubated at 4°C for 5 min with rotation and then centrifuged at 3,000 rpm for 2 min at 4°C. To elute the immunocomplexes from the beads, 300 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) and 12 µl 5 M NaCl were added to each tube containing the beads and 20 µl 5 M NaCl was added to the inputs. To reverse the crosslinks between proteins and chromatin, the ChIP samples and inputs were incubated overnight at 65°C. The residual proteins were degraded by adding 1 µl of 20 mg/ml proteinase K, 10 µl 0.5 M EDTA and 20 µl 1 M Tris pH 6.5 and incubating at 45°C for 3 hours. Every 30 minutes the tubes were briefly shaken on a vortex.

To verify the results of the ChIP experiment, the experiment was performed twice. The first ChIP analysis was carried out with one crosslinked sample (0.3 g) and the second analysis was carried out with six crosslinked samples.

**DNA purification and PCR:**

An equal volume of phenol was added to the samples followed by centrifugation at 2,000 rpm for 5 min at 4°C. Taking the upper layer, an equal volume of chisam (24:1 chloroform:isolamylalcohol) was added. The solution was mixed and centrifuged for 3 min at 14,000 rpm at 4°C. The upper layer was taken and 1/10 volume of 3 M NaOAc, 4 µl glycogen and 2 volumes of 100% ethanol were added. The samples were kept at -20°C overnight. The DNA was pelleted by centrifuging for 15 min at 14,000 rpm at 4°C. After removing the supernatant, the tubes were washed with 70% ethanol and spun for 2 min at 14,000 rpm. Then, the pellet was dried and redissolved in 30 µl (50 µl for inputs) 10 mM Tris (pH 7.0). To test if the purified DNA contained the BCTV chromatin, PCR was carried out, using specific primers for amplification of the *CP* gene.

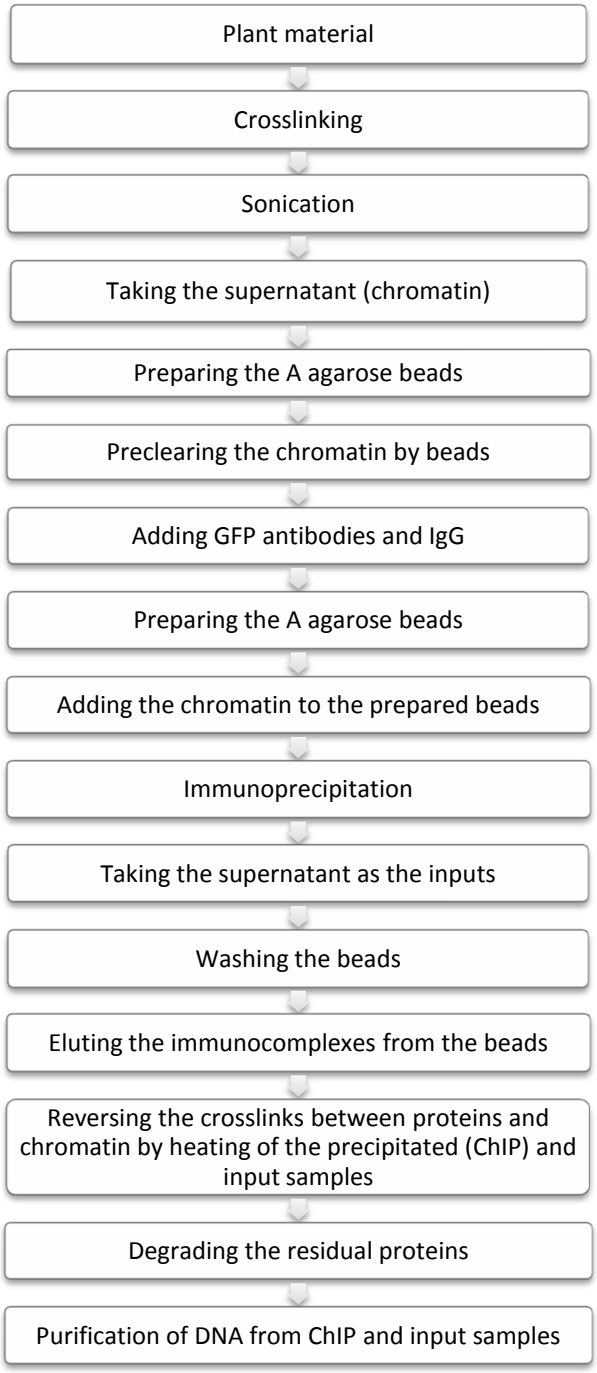


**Fig. 4.** The ChIP steps in this study

# RESULTS:

Approximately 15-20 days after inoculation with BCTV, most of the transgenic GFP-TFL2 plants and Col ecotype showed mild symptoms of virus infection such as leaf curling and necrosis (Fig. 5 and 6). However, no obvious symptom differences were detected in *tfl2-1* and *tfl2-2* plants inoculated with BCTV compared to non-inoculated mutants and the inoculated plants survived as long as the control.

**Fig. 5.** Viral symptoms in transgenic GFP-TFL2 Arabidopsis 20 days post-inoculation with *Beet curly top virus*.

**Fig. 6.** Viral symptoms in transgenic GFP-TFL2 Arabidopsis (black arrow) compared with control plants (blue arrow). The picture was taken 20 days post-inoculation with *Beet curly top virus*.

Twenty days post-inoculation, 15 transgenic GFP-TL2 plants, 15 Col ecotype and 10 *tfl2-1* and 10 *tfl2-2* mutant plants (except for the second experiment) were selected randomly and tested by PCR to detect the presence of virus (Table 1). In each experiment, at least 50 % of the plants, which were tested by PCR, showed the expected positive band with a size of around 500 bp, indicating amplification of the *CP* gene. No band was observed for the mock-inoculated plants or the negative controls, which were tested by PCR (Fig. 7-9). The DNA concentration of the purified PCR products was on average 123 ng/µl.

**Table 1.** The results of inoculation of transgenic GFP-TFL2 Arabidopsis, Col ecotype and mutant Arabidopsis with *Beet curly top virus* in three experiments.

| | **Number of plants tested by PCR** | **# of positive samples in 1st experiment** | **% of positive samples 1st experiment** | **# of positive samples in 2nd experiment** | **% of positive samples 2nd experiment** | **# of positive samples in 3rd experiment** | **% of positive samples 3rd experiment** |
|---|---|---|---|---|---|---|---|
| **Transgenic plants** | 15 | 12 | 80 | 10 | 67 | 11 | 73 |
| **Col ecotype** | 15 | 14 | 93 | 9 | 60 | 8 | 53 |
| ***tfl2-1*** | 10 | 8 | 80 | - | - | 6 | 60 |
| ***fl2-2*** | 10 | 6 | 60 | - | - | 5 | 50 |

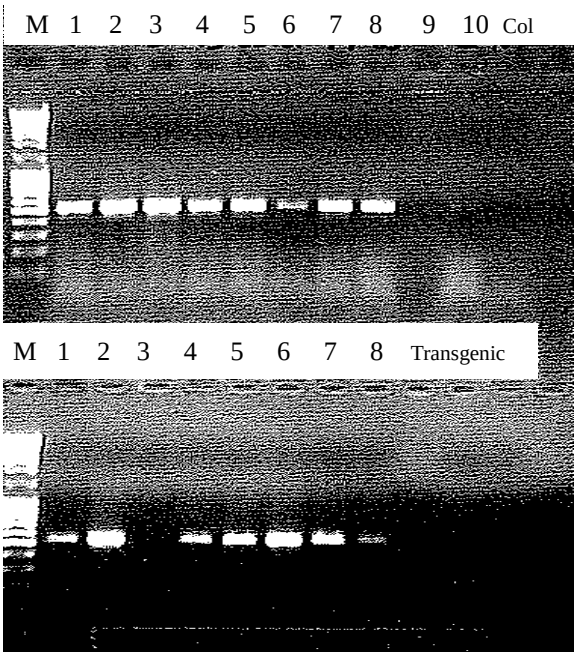


**Fig. 7.** Amplification of *CP* fragment of *Beet curly top virus* from Arabidopsis Col (upper row) and TFL2-GFP transgenic plants (lower row), 20 days post-inoculation. M is GeneRuler™ 100bp DNA ladder plus marker. Lane 9 in the upper gel is a negative control (healthy plant). Lane 10 is a negative control for PCR.

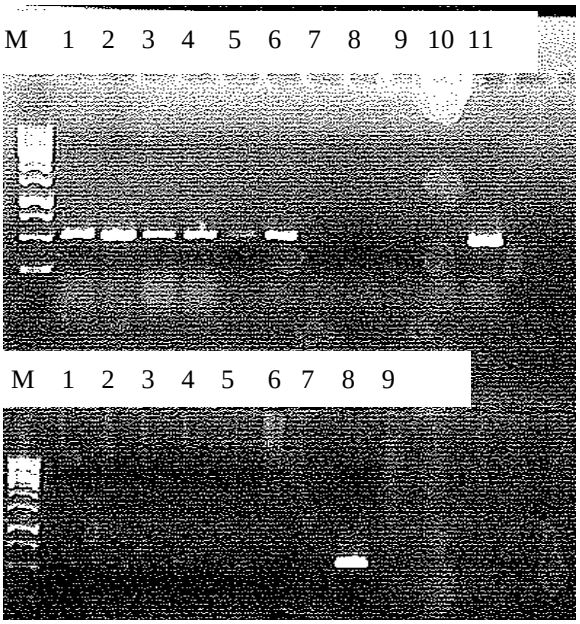


**Fig. 8.** The result of amplification of *CP* fragment of *Beet curly top virus* from transgenic TFL2-GFP Arabidopsis (lanes 1 to 11 in upper gel) and from mock and negative control transgenic samples (lanes 1 to 7 in lower gel). M is GeneRuler™ 1kb DNA ladder marker. In the lower gel, lanes 1 and 2 are samples inoculated with *Agrobacterium tumefaciens* containing empty vector, 3 and 4 inoculated with *Wheat dwarf virus*, 5 to 7 samples stabbed with needle, 8 is a transgenic sample agroinoculated with BCTV and 9 is a negative control for PCR.

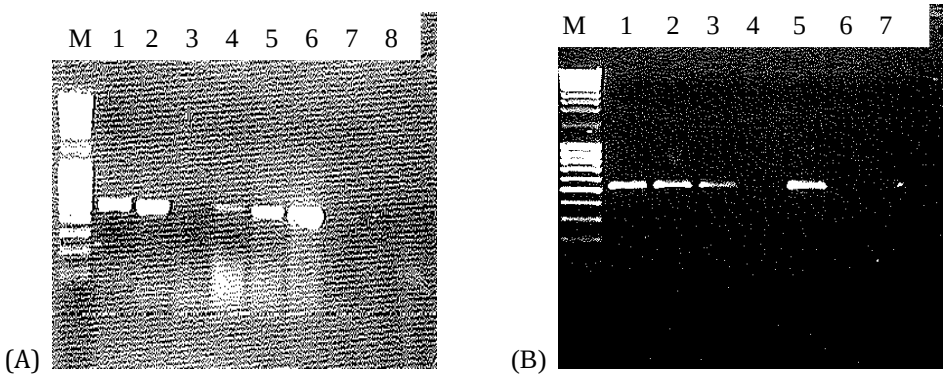


**Fig. 9.** Amplification of *CP* fragment of BCTV from *tfl2-1* (A) and *tfl2-2* (B), 20 days after agroinoculation with BCTV. M is 100bp DNA ladder plus marker (GeneRuler™). Lane 7 in A and lane 6 in B are negative controls (healthy plants). Lane 8 in A and lane 7 in B are PCR negative controls.

Cloning of the *CP* fragment was carried out for 5 samples. The amplified *CP* fragments were purified and ligated into the pGEM-T Easy vector. After digesting the plasmids with *Eco*RI, which cuts twice in pGEM-T and not within the *CP* gene, two bands were observed, a band with the size of the vector (around 3 kb) and a band with the size of the *CP* fragment (around 0.5 kb) (Fig. 10). Three cloned fragments were sequenced. Sequence comparison using Blastn showed that the cloned fragments were 100 % identical to the *CP* gene of BCTV-California (Logan) with the accession number M24597.

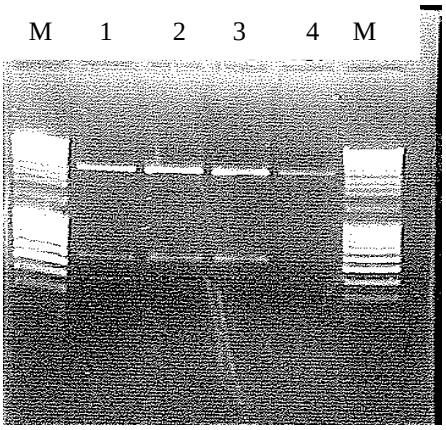


**Fig. 10.** Digestion of pGEM-T with *Eco*RI. The upper band is the pGEM-T vector. The lower band is the inserted *CP* fragment of BCTV isolated from a transgenic (lane 1), a Col ecotype (lane 2), a *tfl2-1* (lane 3) and a *tfl2-2* (lane 4) plant. M is 100bp DNA ladder plus marker (GeneRuler™).

Using RCA, the complete genome of BCTV (about 3.0 kb) was amplified from six inoculated plants (two transgenic, two Col ecotype and one from each mutant) (Fig. 11), suggesting the presence of the circular BCTV genome in the inoculated Arabidopsis plants. However, the DNA isolated from a non-inoculated plant also showed an amplification product (Fig. 12). Different restriction enzymes, which have a single restriction site in the BCTV genome, such as *Bgl*II and *Sac*I, were used to release the full-length unit of the viral genome (around 3 kb). However, the amplified DNA was not cut with any of these enzymes (Fig. 13). Also, digestion of the amplification product was tested by enzymes that have three cutting sites in the BCTV genome such as *Ssp*I, but no band was obtained (Fig. 14).

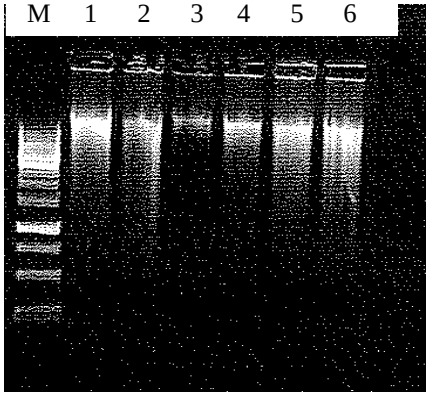


**Fig. 11.** Amplification of the complete circular BCTV genome isolated from transgenic Arabidopsis (lane 1 and 2), Col ecotype (lane 3 and 4), *tfl2-1* (lane 5) and *tfl2-2* (lane 6). M is 1kb DNA ladder marker (GeneRuler™).

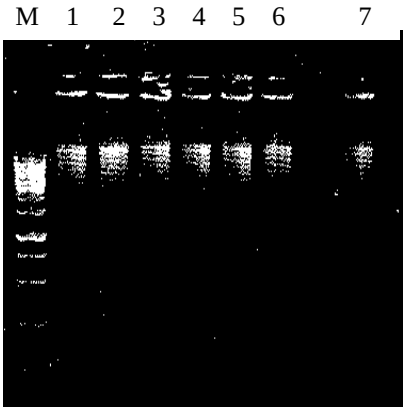


**Fig. 12.** Amplification of the circular DNA isolated from transgenic Arabidopsis (lane 1 and 2), Col ecotype (lane 3 and 4), *tfl2-1* (lane 5), *tfl2-2* (lane 6) and a non-inoculated Col ecotype Arabidopsis (lane 7). M is 1kb DNA ladder marker (GeneRuler™).

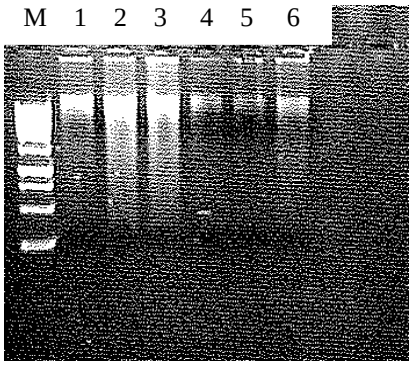


**Fig. 13.** Restriction of the amplified circular DNA by *Bgl*II (lanes 1 - 3) and *Sac*I (lanes 4 - 6), which have a single restriction site in the genome of *Beet curly top virus*. M is 1kb DNA ladder marker (GeneRuler™). Lanes 1 and 4 are the amplified DNA from inoculated transgenic Arabidopsis, lanes 2 and 5 from Col ecotype, lanes 3 and 6 from a *tfl2-1*.

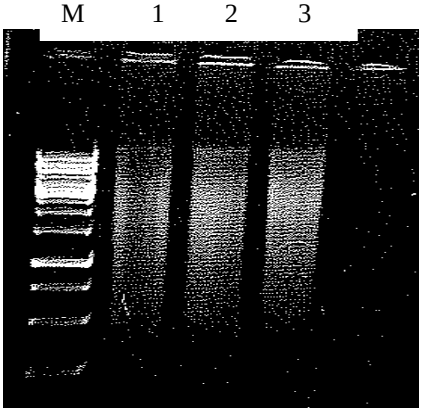


**Fig. 14.** Restriction of the amplified circular DNA by *Ssp*I, which has three cutting sites in the genome of *Beet curly top virus*. M is 1kb DNA ladder marker (GeneRuler™). Lane 1 is the amplified DNA isolated from an inoculated transgenic Arabidopsis, lane 2 from a Col ecotype and lane 3 from a *tfl2-1* plant.

The ChIP experiment was carried out two times to investigate whether the TFL2 protein interacts with the viral chromatin. After cross-linking, the viral DNA was sonicated to obtain an average size of 500 bp. Following immunoprecipitation with antibodies specific for the GFP-tag of TFL2, the obtained DNA fragments were tested by PCR, using the viral *CP* primers to check for the presence of viral DNA in the precipitated DNA and also in the inputs. The DNA concentration of the ChIP products is shown in Tables 2 and 3.

Using PCR test for the first ChIP analysis, the chromatin samples precipitated by GFP and also IgG antibodies showed a PCR product with a size of about 500 bp (lanes 2 and 4 in Fig. 15). However, no band was obtained for the input samples (Fig. 15). After the second ChIP analysis and PCR, among six chromatin samples precipitated by GFP antibody (GFP ChIP samples), two samples showed a thin band, indicating amplification of the *CP* gene (Fig. 16. Lanes 4 and 5). Also, among six IgG ChIP samples, two samples (lanes 9 and 12) showed positive bands. Furthermore, all the five input samples tested by PCR showed amplification of the *CP* gene where two of them were GFP inputs (lanes 13 and 14) and three were IgG input samples (lanes 15, 16 and 17).

Furthermore, to check if the chromatin was sheared into an appropriate fragment size (250 to 750 bp), 10 µl from each supernatant was taken after sonication. These samples were de-crosslinked along with the ChIP samples and run on the agarose gel. However, no clear chromatin band was observed on the gel and the chromatin size could not be checked (not shown).

**Table 2.** The concentration of precipitated DNA (GFP and IgG ChIP) and inputs after the first analysis.

| Sample | DNA concentration (ng/µl) |
|---|---|
| **GFP ChIP** | 76 |
| **IgG ChIP** | 21 |
| **GFP- Input** | 429 |
| **IgG-Input** | 240 |

**Table 3.** The concentration of precipitated DNA (GFP and IgG ChIP) and inputs after the second analysis (an average from six samples).

| Sample | DNA concentration (ng/µl) |
|---|---|
| **GFP ChIP** | 31 |
| **IgG ChIP** | 5 |
| **GFP-Input** | 97 |
| **IgG-Input** | 21 |
| **Input after sonication** | 276 |

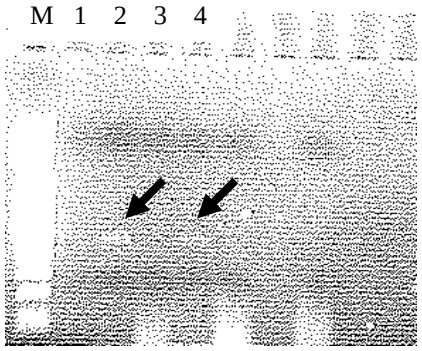


**Fig. 15.** PCR amplification of *CP* fragments after the first ChIP analysis. M is 100bp DNA ladder plus marker (GeneRuler™ ). Lane 1 is the IgG input sample, 2 is the IgG ChIP sample, 3 is the GFP input sample and 4 is the GFP ChIP sample. The arrows show the PCR products with the size of 500 bp (the size for the amplification product of the *CP* gene).

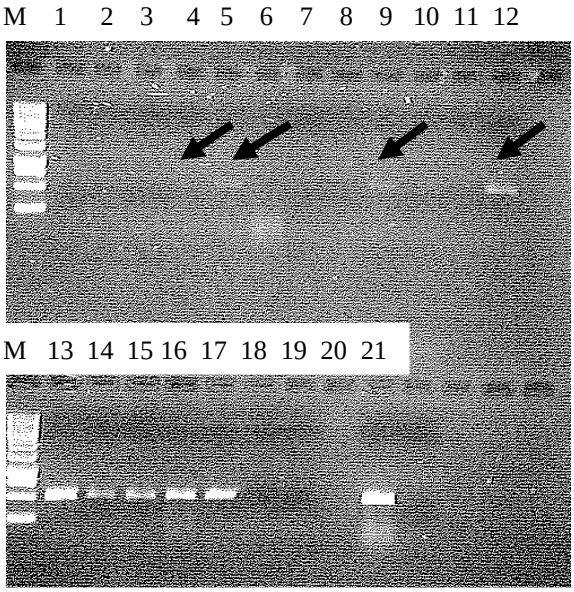


**Fig. 16.** PCR amplification of *CP* fragments after the second ChIP analysis. M is 1kb DNA ladder marker (GeneRuler™). Lanes 1 to 6 show the GFP ChIP samples. Lanes 7 to 12 show the IgG ChIP samples. Lanes 13 and 14 are the GFP inputs. Lanes 15 to 17 are IgG input samples. Lanes 18 and 19 are the samples taken after sonication. Lane 20 is negative control for PCR. Lane 21 is a positive control for PCR (DNA isolated from an infected plant). The PCR product is 500 bp (the size for the amplification product of the *CP* gene). The arrows show the PCR product of GFP and IgG ChIP samples.

# DISCUSSION:

To study the possible interaction of BCTV chromatin and TFL2, one part of the experiment was planned to monitor the development of virus symptoms in transgenic TFL2-GFP Arabidopsis, Col ecotype and *tfl2* mutant plants. From each group of plants, some were mock inoculated to be compared with the plants inoculated with BVTV. The first viral symptoms on transgenic and Col plants were observed around 15 to 20 days after inoculation. In comparison with plants inoculated with WDV, *A. tumefaciens* with empty vector, plants stabbed by needle, and control plants (plants with no inoculation), transgenic and Col plants infected by BCTV showed a range of mild symptoms including leaf curling, smaller rosette leaves and necrosis. Considering the hypothesis that TFL2 interacts with BCTV chromatin and plays a role in silencing of this virus, it was expected to see severe viral symptoms in *tfl2* mutant plants. However, no typical virus symptoms were observed in these *tfl2* plants, indicating that Arabidopsis plants are capable of countering BCTV even in the absence of TFL2.

At the molecular level, the presence of BCTV in the agroinoculated plants was verified by PCR amplification of the viral *CP* gene. Sequencing the amplified *CP* fragments after cloning into *E. coli* bacteria allowed us to verify that the plants were infected with BCTV. The *CP* sequences showed 100% identity to BCTV-California (Logan), with which the plants had been agroinoculated. This result indicates that agroinoculation of plants with BCTV using entomological needles was carried out successfully. Furthermore, the high number of plants, which were virus positive in the PCR test, suggested that this method for BCTV inoculation is effective for Arabidopsis.

To detect the circular BCTV genome in the inoculated plants, the RCA method was used. RCA products were obtained for the tested inoculated plants and surprisingly in a non-inoculated plant, which was used as the negative control. To prove the presence of the BCTV genome in the RCA products, different restriction enzymes were used. However, the RCA products were not cut with any of them. In some cases, the result from restriction of RCA products was similar to the digestion of genomic plant DNA (Fig. 14). The RCA/RFLP results suggest that a circular

plant DNA might have been amplified, which could be a mitochondrial plasmid (Homs et al., 2008) and not the BCTV genome.

To investigate the interaction between TFL2 and BCTV chromatin, two sets of samples were analysed with the ChIP method. The ChIP analysis was based on immunoprecipitation of the GFP:TFL2:DNA complex using antibodies against GFP. To determine whether the chromatin, which was precipitated by antibodies, contained viral DNA, PCR was run using *CP* primers. To provide support for the hypothesis that TFL2 interacts with BCTV chromatin, a PCR product from this DNA sample was expected.

To estimate the reliability of the ChIP data, two types of control samples were included in the experiments: the input sample and also the sample with non-specific antibody. The input sample indicates the presence and amount of the chromatin fragment of interest in the ChIP reaction. In this study the input was the supernatant taken from the chromatin after precipitation using antibodies. Then following de-crosslinking, the DNA of input samples was isolated and used in a PCR test. This isolated chromatin contained the viral DNA fragments, which were not bound to the protein A agarose beads and were not precipitated. Even in the case when TFL2 has bound to the viral chromatin and the binding efficiency of GFP antibody to the complex is high enough, considering the amount of protein A agarose beads in the tubes, obviously not all the DNA-protein complexes will be captured by the beads. So the input DNA, as a positive control, should yield *CP* PCR products. In this experiment, in addition to the inputs, another control sample was the chromatin to which a non-specific antibody (IgG antibody) instead of the specific antibody (GFP) was added. These control samples (IgG ChIP) were treated in the same way as the ChIP reaction samples and used to determine whether the precipitation was specific for the bound chromatin of interest. So, it was not expected to get PCR products for them.

As shown in Fig. 15, the first ChIP analysis resulted in a PCR product with the size of the *CP* fragment (about 500 bp) for the chromatin precipitated with GFP and IgG antibodies. However, no band was obtained for the GFP and IgG inputs. The second ChIP analysis also resulted in amplification of the *CP* fragment for some of the samples precipitated with GFP and IgG. Furthermore, in this ChIP experiment, the GFP and IgG inputs yielded the expected PCR

products (Fig. 15). Obtaining a PCR product for the ChIP sample precipitated by GFP antibody might prove that TFL2 was cross-linked to the viral chromatin properly and that this complex was captured by the beads when binding to GFP antibody. Although, ideally, it is not expected to obtain a band for the DNA sample precipitated with a non-specific antibody, PCR bands were observed for some of the IgG ChIP samples as well. Obtaining a PCR product for the IgG ChIP sample could be acceptable if it is quantitatively proven that the amount of DNA fragment of interest in the ChIP sample with non-specific antibody (IgG) is much less than in the ChIP sample with the specific antibody (GFP). Otherwise, it could be evidence for that the precipitation is non-specific.

On the other hand, in the first ChIP experiment (Fig. 15), not obtaining any band for the input samples (as a positive control) indicates the absence of viral genome in the supernatant taken from the chromatin after precipitation using antibodies. However, the DNA concentration of these input samples is much higher than the ChIP samples (Table 1). Considering the presence of viral genome in the precipitated chromatin, it might be concluded that the viral genome in inputs was mistakenly removed during the experiment or that there is no association between BCTV chromatin and TFL2. Taken together, the reliability of this ChIP analysis based on the results obtained from the control samples cannot be ensured.

As shown in Fig. 16, the bands obtained for the inputs in the second experiment illustrate the high amount of viral genome in the supernatant taken after precipitation. Also the bands of GFP ChIP samples could prove the presence of interaction between TFL2 and viral chromation. However, not all the GFP ChIP samples showed the bands. This can suggest that either TFL2 was not cross-linked to the viral chromatin as a result of low efficiency of the cross-linking step or the complex was not precipitated by GFP antibody. Different factors can affect the immunoprecipitation procedure such as not shearing the DNA into the proper size in the sonication step or low efficiency of the antibody's binding to the complex.

In summary, the data obtained from the second ChIP analysis, compared to the first one, gives more reliable insight towards the interaction between TFL2 and the viral chromatin, since the results of the positive controls were as expected.

Taken together, the results of the inoculation experiments suggest that TFL2 does not play an important role in the defence against BCTV, since the *tfl2* mutants did not show hypersusceptibility to the virus. However, it is possible that in the absence of TFL2 some other proteins are activated to counter BCTV. In this study, the ChIP analysis could not support the hypothesis that TFL2 interacts with BCTV chromatin and obviously the experiment needs to be repeated to verify if there is an association between this protein and geminiviral chromatin. Since precipitation of chromatin in the ChIP method is not completely specific, the ChIP data should be analysed quantitatively for a more detailed conclusion.

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**Department of Plant Biology and Forest Genetics**
**SLU**
**BOX 7080**
**75007 Uppsala, Sweden**